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PRINCIPAL INVESTIGATOR: Dr. Gina Forster

CONTRACTING ORGANIZATION: The University of South Dakota
Vermillion, SD 57069

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Dr. Gina Forster

E-Mail: gforster@usd.edu

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14. ABSTRACT
Previous correlational studies suggest that mild traumatic brain injury (TBI) is associated with an increase in the prevalence of anxiety disorders, including posttraumatic stress disorder (PTSD). By using a relevant animal model, this research demonstrates that mild TBI can result in increased fear learning and anxiety states that mimic the symptoms of generalized anxiety disorder and PTSD. Furthermore, this research suggests that heightened fear and anxiety states following mild TBI may result from alterations to cell death and neuronal number in limbic brain regions such as the hippocampus and amygdala. Recent data generated from this project also suggests that experiencing psychosocial stress concurrent with mild brain injury results in serotonin dysfunction in the limbic system (particularly the hippocampus) and exacerbates anxiety states. Therefore, changes to limbic system function may underlie anxiety states that manifest following mild TBI and stress. Overall, the current rodent model of mild TBI appears to be a useful model to explore the neurobiological link between mild TBI and anxiety states, to allow better understanding of the consequences of war injuries like mild TBI, and to explore how such injury could be treated to prevent long-term disability.

15. SUBJECT TERMS
Mild traumatic brain injury; Posttraumatic stress disorder; Neurobiology of anxiety disorders

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Introduction:

The greater incidence of mild traumatic brain injury (TBI) arising from recent military conflicts could be in part responsible for the increasing occurrence of posttraumatic stress disorder (PTSD) in US troops (Kennedy et al., 2007; Gaylord et al., 2008; Hoge et al., 2008). Brain regions susceptible to shearing and bruising during a mild TBI incident overlap with those brain regions that process emotion and regulate behavioral and physiological responses to stressors (Kennedy et al., 2007; Bryant, 2008). These convergent brain areas include the medial prefrontal cortex (mPFC) and the temporal lobe including the amygdala and hippocampus (Kennedy et al., 2007; Bryant, 2008). Alterations to serotonergic function have been implicated in anxiety disorders including PTSD (Stam, 2007; Lowry et al., 2008). Serotonergic activity in the amygdala is related to the onset while serotonin release in the mPFC is related to the cessation of fear behavior in an animal model (Forster et al., 2006). Thus, amygdala hyperfunction/mPFC hypofunction observed in PTSD (Milad et al., 2006; Rauch et al., 2006; Stam, 2007) may be a result of altered serotonin activity, which could arise from mild trauma to these regions. Furthermore, serotonin activity in relation to fear and anxiety states is regulated by corticotrophin-releasing factor (CRF), released in response to physical and psychological stressors (Forster et al., 2006; Lukkes et al., 2009a). Severe or chronic stressors increase CRF levels and the levels of CRF receptors, thus affecting regulation of serotonin activity and leading to heightened anxiety states (as reviewed by Stam, 2007; Lukkes et al., 2009a). Therefore, both CRF and serotonin represent important potential targets for the treatment of PTSD (Stam, 2007), but the impact of mild TBI on these systems is unknown. Therefore current research addresses the following Objectives:

- Objective 1: Establish whether mild TBI results in neural death or altered serotonin/CRF function within limbic brain regions that are implicated in the symptomology of posttraumatic stress disorder (PTSD).
- Objective 2: Relate altered function of limbic brain regions following mild TBI with psychobehavioral and neuroendocrine symptoms of PTSD.

Body:

The Milestones within the approved Statement of Work for this award are outlined in Table 1 below.

Table 1: Project Objectives and Approved Milestones

	Quarter 1 Year 1	Quarter 2 Year 1	Quarter 3 Year 1	Quarter 4 Year 1	Quarter 1 Year 2	Quarter 2 Year 2
Objective 1: Establish whether mild TBI results in neural death or altered neural/neurochemical function within limbic brain regions that are implicated in the symptomology of PTSD.						
Task 1: Animal IACUC approved	University of South Dakota					
Task 2: Measure markers of neuronal death susceptibility, neuronal number and apoptotic neurons.	Begin to collect samples end of this quarter.	Collect samples and begin analysis.	Analyze samples.			
Task 3: Measure serotonin.			Collect Samples	Analyze samples.		
Task 4: Measure CRF and receptors.				Samples Collected.	Analyze samples.	
Task 5: Initial Dissemination of Results			Conference Presentation			
Objective 2: Relate altered function of limbic brain regions following mild TBI with psychobehavioral and neuroendocrine symptoms of PTSD.						
Task 1: Fear conditioning and extinction testing				Begin behavioral testing.	Continue behavioral testing.	Finish behavioral testing beginning of this quarter. Data Analyzed.
Task 2: Dissemination of Results				Write manuscripts on molecular data.	Write manuscripts on neurochemical data.	Write manuscripts on behavioral data. Conference presentation.

Objective 1 Task 1 - Obtain IACUC approval from the University of South Dakota to perform the research.
IACUC approval from the University of South Dakota, and approval from the USAMRMC Animal Care and Use Research Office were both obtained prior to the award starting. This allowed research to begin immediately upon receiving the award on 9/1/10.

Objective 1 Task 2 – Collect and analyze samples related to neuronal number, neuronal death and apoptosis following mild TBI.
The investigators collected neural tissue from young adult male rats that were subjected to weight drop (mild TBI) either 4 days or 9 days following the mTBI procedure (please see Meyer et al., 2012 in the Appendix for detailed methods). There was no gross morphological damage incurred as a result of the mild TBI. Also, tests of motor function were conducted prior to tissue collection to ensure that the weight drop did not produce a motor deficit that would indicate a more moderate to severe injury (please see Figure 7 of Meyer et al., 2012 in the Appendix). Brains were sectioned and stained to identify neurons for neuronal cell counts, and also stained using the TUNEL method to identify apoptotic cells. Regions of the limbic system thought to mediate anxiety states were analyzed using stereological methods (please see Meyer et al., 2012 in the Appendix for detailed methods). These included the medial prefrontal cortex, amygdala subregions (medial, central and lateral), and dorsal and ventral subregions of the hippocampus (CA1, CA3 and dentate gyrus). Statistical analysis of the neuronal cell counts suggested selective neuronal loss in the CA1 region of the dorsal hippocampus, and increased cell number in the medial and lateral subregions of the amygdala 9 days following mild TBI (please see Table 3 and Figures 2 & 3 of the Meyer et al., 2012 in the Appendix). These alterations to cell numbers appear to be due to changes in apoptosis in these regions. For example, significantly increased density of apoptotic cells in the CA1 region of the dorsal hippocampus was observed 4 days following mild TBI (Figure 6 of Meyer et al., 2012 in the Appendix), suggesting that the decreased neuronal number observed in this region at 9 days following mild TBI was due to increased programed cell death following the injury. On the other hand, significantly decreased density of apoptotic cells was observed in the medial and lateral subregions of the amygdala 4 and 9 days following mild TBI (Figure 5 of Meyer et al., 2012 in the Appendix), suggesting that increased neuronal numbers in these regions at 9 days following mild TBI were due to decreased programmed cell death as a result of mild TBI. Overall, these findings imply that mild TBI differentially activates pro-apoptotic mechanisms in the hippocampus and pro-survival molecular pathways in the amygdala, which should be tested by future research (please see the Discussion of the Meyer et al., 2012 in the Appendix for a detailed discussion of this point). A grant application for a DoD Psychological Health/Traumatic Brain Injury Research Program Applied Neurotrauma Research Award (Funding Opportunity Number: W81XWH-11-PHTBI-ANRA) was submitted in January 2012 to examine pro-apoptotic and pro-survival mechanisms in the limbic system following mTBI, based on the data collected under the current award.

Objective 1 Task 3 – *Collect and analyze samples to measure serotonin levels in the limbic system of rats exposed to mild TBI with or without psychosocial stress.*
For this particular objective, brain tissue from rats in their home cages (at rest) was obtained seven days following one of four treatments (n = 10-12 per treatment group): 1) control handling and sham surgery 2) control handling and mild TBI surgery 3) social defeat stress and sham surgery and 4) social defeat stress and mild TBI surgery. Social defeat stress was used as the psychosocial stressor in these experiments and was performed immediately prior to the mild TBI or sham surgery, using procedures established in the laboratory of the Co-investigator, Dr. Watt (e.g. Watt et al., 2009). Brains from these rats were been sectioned to allow microdissection of the regions of interest (prefrontal cortex, dorsal hippocampus, ventral hippocampus, central, basolateral and medial amygdala, and dorsal raphe nucleus – the serotonin cell body region). The monoamines serotonin, norepinephrine, epinephrine and dopamine, and their precursors and their metabolites were measured from each brain region using HPLC and electrochemical detection (Watt et al., 2009). Following HPLC analysis of the supernatant drawn from the dissected tissue, the tissue content of each sample was analyzed using a Bradford assay, such that the level of monoamine in each sample (pg) was normalized against the amount of tissue in each sample (μg) to provide a pg/μg value for each sample (Watt et al., 2009 and Fig 1 below).

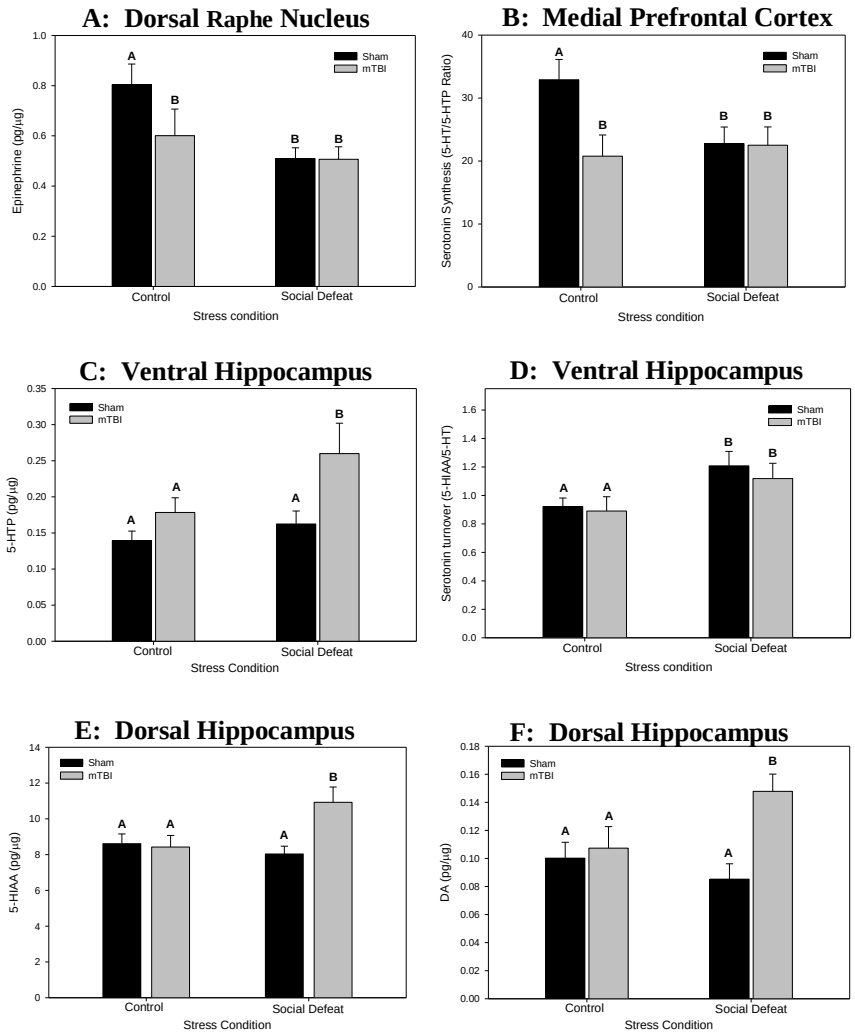

Figure 1: Monoamine levels in limbic regions following mild TBI and/or psychosocial stress (social defeat), 7 days following injury.
Bars with different letters are significantly different from one another ($P < 0.05$). n = 10-12 per group.
5-HT = serotonin; 5-HIAA = serotonin metabolite; 5-HTP = serotonin precursor; DA = dopamine.

We observed that either mild TBI or social defeat stress alone significantly reduced epinephrine levels in the dorsal raphe nucleus (serotonin cell body region), although the combined mild TBI and social defeat stressor did not further impact epinephrine levels in this region (Fig 1A). These findings suggest that epinephrine neurotransmission in the dorsal raphe may be impaired following either brain injury or stress. Since receptors for epinephrine alter the activity of serotonin neurons in the dorsal raphe nucleus (Judge and Gartside, 2006), these findings suggest that altered serotonin activity in terminal regions such as the hippocampus (Fig 1), could arise from reduced activity of epinephrine in the dorsal raphe nucleus. An interesting implication of the finding from the dorsal raphe is that mild traumatic brain injury results in the same changes in the neurochemistry of this brain region as psychosocial stress experience.

Within the medial prefrontal cortex (Fig 1B), rate of serotonin synthesis was significantly reduced by mild TBI and by psychosocial stressor, while mild TBI and psychosocial stressor combined did not further alter serotonin synthesis in this region. The levels of serotonin in the medial prefrontal cortex did not significantly differ between groups ($P > 0.05$), suggesting that at rest, the lower rate of serotonin synthesis does not detrimentally affect stored serotonin levels. However, this data predicts that during time of stress or in an anxiogenic environment when serotonin release, activity and turnover is increased (e.g. Forster et al., 2006), animals exposed to mild TBI and/or psychosocial stress will show serotonin deficits. Importantly, serotonin deficits in the medial prefrontal cortex is associated with increased anxiety (Pum et al., 2009), suggesting that this neurochemical finding in the medial prefrontal cortex could contribute to the increased anxiety seen

following mild TBI and/or psychosocial stress (Fig. 3).

There were two main neurochemical changes observed in the ventral hippocampus as a result of mild TBI and/or psychosocial stress (Fig 1). The levels of serotonin precursor 5-HTP were increased in rats exposed to both mild TBI and psychosocial stress as compared to all other treatment groups (Fig 1C). Higher levels of 5-HTP reflect less conversion of 5-HTP to serotonin (Evans et al., 2009). While serotonin levels in the ventral hippocampus did not differ between groups ($P > 0.05$) at rest, it is expected that during exposure to a stressor or an anxiogenic environment when serotonin release and turnover increases (Barr and Forster, 2011), a serotonin deficit would be revealed. Like the medial prefrontal cortex, lower serotonin in the ventral hippocampus is associated with greater anxiety (see Barr et al., 2010; Barr and Forster, 2011), thus suggesting serotonin dysfunction in the ventral hippocampus of rats exposed to both mild TBI and psychosocial stressor could underlie the greater anxiety states in these rats compared to all other treatment groups. Interestingly, serotonin turnover (a measure of serotonin activity) in the ventral hippocampus at rest was increased by psychosocial stress experienced 7 days previous (Fig 1D), with no effect of mild TBI on this measure. This may indicate a long-term change in serotonin function following psychosocial stress that differs from the effect of mild TBI. It remains to be seen whether increased serotonin turnover at rest 7 days following psychosocial stressor manifests and altered serotonin release during subsequent stress exposure.

Within the dorsal hippocampus, the level of the serotonin metabolite 5-HIAA were increased in rats exposed to both mild TBI and psychosocial stress as compared to all other treatment groups (Fig 1D). Higher level of 5-HIAA is an index of increased conversion of serotonin to 5-HIAA, typically a result of increased serotonin release (Moore and Johnson, 1982; Shannon et al., 1986). Therefore, the combined exposure of mild TBI and psychosocial stressor results in increased serotonin release in the dorsal hippocampus at rest, when measured 7 days following the insult. Increased postsynaptic activation of serotonin receptors in the dorsal hippocampus increases anxiety behavior in rats (Romaniuk et al., 2001). Therefore, increased serotonin function in the dorsal hippocampus could also contribute to augmented anxiety behavior in rats exposed to both mild TBI and psychosocial stress, as compared to either insult alone (Fig 3). Rats exposed to both mild TBI and psychosocial stress also showed greater dopamine levels in the dorsal hippocampus (Fig 1E) as compared to all other treatment groups. Postsynaptic effects of dopamine on receptors in the dorsal hippocampus mediate long-term fear memory (Rossato et al., 2009). Thus, the effects of mild TBI and psychosocial stress on dopamine in the dorsal hippocampus suggest that these insults combined would enhance long-term fear memory, which should be tested by future research.

Overall, the neurochemical survey of the limbic system of rats exposed to mild TBI and/or psychosocial stress demonstrates several areas of serotonin dysfunction, that may manifest as increased anxiety in anxiogenic or stressful environments. To directly test the relationship between stress, anxiety and serotonin release, a grant application for a DoD Psychological Health/Traumatic Brain Injury Research Program Applied Neurotrauma Research Award (Funding Opportunity Number: W81XWH-11-PHTBI-ANRA) was submitted in January 2012, using the rat model developed by this current proposal as the basis for study.

Objective 1 Task 4 – *Collect samples to measure CRF receptor levels in the limbic system of rats exposed to mild TBI with or without psychosocial stress.*

The regulation of serotonin activity throughout the limbic system during stress is mediated by corticotropin-releasing factor (CRF) receptors, located in the serotonin cell body group – the dorsal raphe nucleus (Forster et al., 2006; 2008; Lukkes et al., 2008; Mo et al., 2009; Scholl et al., 2010). Furthermore, up-regulation of the CRF type 2 receptor (CRF_2) in the dorsal raphe nucleus has been associated with increased anxiety states in other animal models (Pringle et al., 2008; Lukkes et al., 2009b; Lukkes et al., 2009c; Vuong et al., 2010; Bledsoe et al., 2011). Thus, the increased anxiety states following psychosocial stress and mTBI (Fig 3) may relate to increased expression of CRF_2 receptors in the dorsal raphe nucleus. To study the expression of the three types of CRF receptors (CRF_1, CRF_2 and CRF-binding protein), brains were obtained 7 days following one of four treatments (n = 10 rats per treatment group): 1) control handling and sham surgery 2) control handling and mild TBI surgery 3) social defeat stress and sham surgery and 4) social defeat stress and mild TBI surgery. Brains were sectioned to allow microdissection of the dorsal raphe nucleus, and CRF receptor levels were measured using western blot methods established in the PI' s laboratory (Lukkes et al., 2009b).

There was no effect of mild TBI or psychosocial stress on the levels of CRF binding protein (CRF-BP) or CRF_1 receptors in the dorsal raphe nucleus (Fig 2A-B). This finding matches those previous studies

examining CRF receptor levels in the dorsal raphe, showing no effect of stress or anxiogenic treatments on the levels of these receptors (Pringle et al. ,2008; Lukkes et al., 2009b). However, both mild TBI and psychosocial stress decreased CRF_2 receptor levels in the dorsal raphe nucleus (Fig 2C). More surprisingly, the combined insults of mild TBI with psychosocial stress appeared to reverse the effects of each insult alone, as CRF_2 levels were similar to controls in the mild TBI with psychosocial stress group. As increased CRF_2 receptor levels in this region have traditionally been associated with increased anxiety, and reducing CRF_2 receptor activity decreases anxiety (Vuong et al., 2010; Bledsoe et al., 2011), it is likely that the changes observed in CRF_2 receptor levels in the dorsal raphe nucleus do not underlie the increased anxiety states observed following mild TBI with and without psychosocial stress (Fig 3). Future work could decipher which neuronal types in the dorsal raphe nucleus (serotonergic or non-serotoninergic) exhibit decreased CRF_2 receptor expression, which would clarify the impact of the reduced CRF_2 receptor level expression on serotonergic function and behavior.

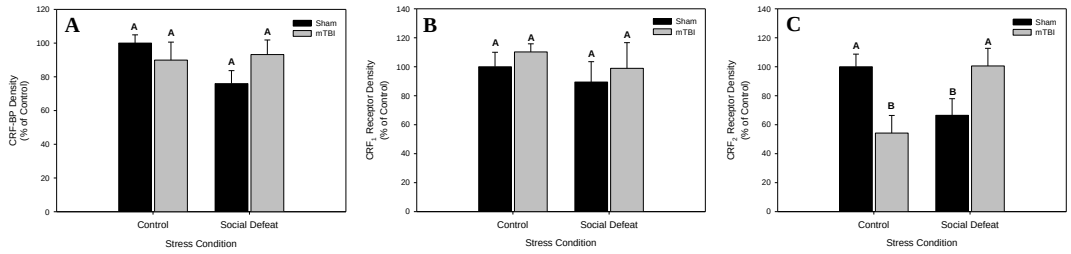

Figure 2: CRF receptor levels in the dorsal raphe nucleus following mild TBI and/or psychosocial stress (social defeat), 7 days following injury.
Bars with different letters are significantly different from one another ($P < 0.05$). n = 10 per group.

Objective 1 Task 5: - *Initial dissemination of results via presentation at a scientific conference.*
The investigators (Forster, Watt and Meyer) attended the annual Society for Neuroscience meeting (San Diego, November 13-17th, 2010). Our preliminary research findings (particularly those from Objective 1 Task 2) were presented by Dr. Forster and Ms. Meyer in the "Animal Models of Traumatic Brain Injury" session of this meeting. The presentation was titled: Meyer, D.L., Davis, D., Barr, J.L., Manzerra, P., and Forster, G.L. (2010). Neuronal loss within the limbic system in a rat model of closed mild traumatic brain injury. *Society for Neuroscience Abstracts*, 467.17.

Objective 2 Task 1: - *Relate altered function of limbic brain regions following mild TBI with fear/anxiety behaviors.*

Decreased neurons in the dorsal CA1 region observed in experiments of Objective 1 suggested that mild TBI may alter contextual fear conditioning (Hunsaker and Kesner, 2008). Furthermore, increased amygdala neuronal counts observed in experiments of Objective 1 may predict increased fear conditioning and generalized anxiety states (Goosens and Maren, 2001; Rauch et al., 2006). To test the functional consequences of the neuronal changes observed following mTBI, rats were subjected to control or mild TBI procedures and were tested either for contextual fear conditioning and extinction or for generalized anxiety behavior (please see Meyer et al., 2012 in the Appendix for methods details). As predicted by the neural changes seen following mild TBI, rats subjected to mild TBI showed heightened contextual fear conditioning (Figure 7 of Meyer et al., 2012 in the Appendix) that could not be explained by heightened nociception (Figure 7C of Meyer et al., 2012 in the Appendix). Furthermore, rats subjected to mild TBI also showed increased generalized anxiety-like behaviors (Figure 8 of Meyer et al., 2012 in the Appendix). Together, these data suggest discrete changes in neural numbers in the limbic system following mild TBI, which are reflected by heightened conditioned fear and anxiety states. Overall, this animal model appears to represent a good tool to assess the neurobiological link between mild TBI and anxiety states.

The behavioral findings following mTBI were then extended by determining whether psychosocial stress (social defeat) at the time of head injury further impacts anxiety states. Rats (n = 10 per group) were randomly assigned to one of four groups, treatments: 1) control handling and sham surgery 2) control handling and mild TBI surgery 3) social defeat stress and sham surgery and 4) social defeat stress and mild TBI surgery. Rats were then tested for anxiety behavior 7 days following treatment, using the elevated plus maze (Meyer et

al., 2012). We found that both mild TBI and social defeat on their own significantly reduced time spent on open arms of the maze, indicative of greater anxiety states (Fig 3A). Interestingly, the combination of mild TBI and psychosocial stressor further increased anxiety states (Fig 3A). Neither treatment affected general activity in the maze (Fig 3B) suggesting that the reduced time spent in open arms of the maze was not simply due to a motor deficit. Overall, these findings suggest that that behaviorally, mild TBI procedures a very similar anxiogenic profile as psychosocial stress, and that the combination of mild TBI with a psychosocial stressor (such as being the recipient of aggression) results in greater psychological effects than either alone. As suggested by data collected for Objective 1 task 3 (Fig 1), serotonergic dysfunction in the limbic system (particularly the hippocampus) may underlie the heightened anxiety induced by mild TBI and psychosocial stress. The mechanism underlying these observations is the focus of a grant application for a DoD Psychological Health/Traumatic Brain Injury Research Program Applied Neurotrauma Research Award (Funding Opportunity Number: W81XWH-11-PHTBI-ANRA), submitted in January 2012.

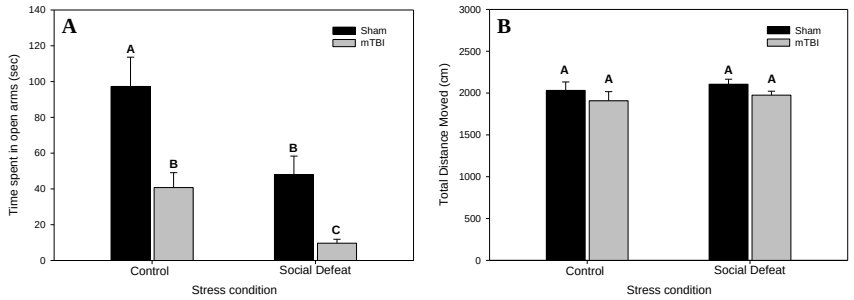

Figure 3: Time spent in open arms and total distance moved in the elevated plus maze, 7 days following mild TBI and/or psychosocial stress (social defeat).
Bars with different letters are significantly different from one another ($P < 0.05$). n = 10 per group.

Objective 2 Task 2: - *Dissemination of results by writing and submitting manuscripts for publication.*
We wrote a manuscript including the molecular/cell count data (Objective 1 task 1) and much of the behavioral results (Objective 2 task 1). This manuscript was accepted for publication in the journal *Experimental Neurology* in March of 2012 and is currently in press (expected to be published online by mid-April 2012). . Please see the Meyer et al. 2012 manuscript included in the Appendix. A poster was presentation at the Faculty for Undergraduate Poster Session at the 2011 Society for Neuroscience Meeting (Washington D.C., November 12-16th , 2011) by an undergraduate student working on this project as a component of their research training: Davis, D.R., Meyer, D.L., Barr, J.L., Manzerra, P., and Forster, G.L. (2011). Increased Anxiety States and Contextual Fear Conditioning in Closed Traumatic Brain Injury Mirroring Post-Traumatic Stress Disorder. *FUN Poster Session,* 40.

The remaining data (serotonin, CRF and behavioral data from Objectives 1 and 2) are included in a manuscript currently being written by the investigators: Forster, G.L., Meyer, D.L., Scholl, J.S., Olson, D., Manzerra, P., and Watt, M.J. Effects of mild traumatic brain injury and psychosocial stress on limbic monoamines and anxiety-like behaviors. To be submitted to: *Brain Research*. A presentation of the same title will be presented by the authors at the annual International Behavioral Neuroscience Society meeting in June of 2012.

Key Research Accomplishments:

- Mild TBI in a young adult male rat model results in increased fear learning and anxiety states.
- Mild TBI also results in alterations to programmed cell death and neuronal numbers in regions of the limbic system that mediate fear and anxiety.
- Alterations to neuronal number following mild TBI do not become apparent until between 5-9 days following the injury.

- Experiencing psychosocial stress concurrent with mild TBI exacerbates anxiety states when compared to mild TBI alone or psychosocial stress alone.
- The effects of mild TBI and psychosocial stress on anxiety states may be mediated by altered serotonin function in the limbic system, particularly in the hippocampus.

Reportable Outcomes:

This research has resulted in the following outcomes:

- A scientific presentation at the 2010 Annual Society for Neuroscience Meeting (San Diego, November 13-17th, 2010): Meyer, D.L., Davis, D., Barr, J.L., Manzerra, P., and Forster, G.L. (2010). Neuronal loss within the limbic system in a rat model of closed mild traumatic brain injury. *Society for Neuroscience Abstracts*, 467.17.
- A scientific presentation at the Faculty for Undergraduate Poster Session, 2011 Society for Neuroscience Meeting (Washington D.C., November 12-16th , 2011): Davis, D.R., Meyer, D.L., Barr, J.L., Manzerra, P., and Forster, G.L. (2011). Increased Anxiety States and Contextual Fear Conditioning in Closed Traumatic Brain Injury Mirroring Post-Traumatic Stress Disorder. *FUN Poster Session,* 40.
- A manuscript accepted for publication in the journal *Experimental Neurology*: Meyer, D.L., Davis, D.R., Barr, J.L., Manzerra, P., and Forster, G.L. Mild Traumatic Brain Injury in the Rat Alters Neuronal Number in the Limbic System and Increases Conditioned Fear and Anxiety-like Behaviors. *Experimental Neurology*, in press.
- A scientific abstract submitted for presentation at the annual International Behavioral Neuroscience Society meeting (June 2012): Forster, G.L., Meyer, D.L., Scholl, J.S., Olson, D., Manzerra, P., and Watt, M.J. Effects of mild traumatic brain injury and psychosocial stress on limbic monoamines and anxiety-like behaviors.
- Mr. Daniel Davies received training and research experiences on this project that have contributed to his honors thesis, a requirement of the Bachelor of Science with honors degree at the University of South Dakota. Mr. Davies has been accepted to the Sanford School of Medicine at the University of South Dakota, to pursue an MD degree.
- A grant was submitted for review in January 2012 for a DoD Psychological Health/Traumatic Brain Injury Research Program Applied Neurotrauma Research Award (Funding Opportunity Number: W81XWH-11-PHTBI-ANRA), based on the work supported by this award. This application is currently under review.
- Ms. Danielle Meyer received training and experience on this project that directly assisted her successful application to Nursing School at the South Dakota State University (SDSU). She is currently in the first year of the fast-track nursing program at SDSU.

In addition (and as requested by the instructions "Technical Reporting Requirements" for final reports), the personnel receiving pay from this research effort were:

Gina Forster, PhD (PI)
Michael Watt, PhD (co-investigator)
Pasquale Manzerra, PhD (co-investigator)
Danielle Meyer, BS (research associate)
Jamie Scholl, MS (research associate)
Daniel Davies (undergraduate researcher)

Conclusions:

Previous correlational studies suggest that mild TBI is associated with an increase in the prevalence of anxiety disorders, including PTSD. By using a relevant animal model, this research demonstrates that mild TBI can result in increased fear learning and anxiety states that mimic the symptoms of generalized anxiety disorder and PTSD. Furthermore, this research suggests that heightened fear and anxiety states following mild TBI may result from alterations to cell death and neuronal number in limbic brain regions such as the hippocampus and

amygdala. Recent data generated from this project also suggests that experiencing psychosocial stress concurrent with mild brain injury exacerbates subsequent anxiety states. Dysfunction of the neurotransmitter serotonin in the limbic system, particularly the hippocampus, was observed following mild brain injury concurrent with psychosocial stress, suggesting that serotonin dysfunction may contribute to heightened anxiety states following these insults. Overall, the current rodent model of mild TBI appears to be a useful model to explore the neurobiological link between mild TBI and anxiety states, and future research should directed at determining the mechanisms by which mild TBI and psychosocial stress interact to exacerbate anxiety states. This will allow better understanding of the consequences of war injuries like mild TBI, and how such injury could be treated to prevent long-term disability.

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Appendix:

A copy of the Meyer et al., 2012 manuscript follows.

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Mild Traumatic Brain Injury in the Rat Alters Neuronal Number in the Limbic System and Increases Conditioned Fear and Anxiety-like Behaviors.

Danielle L. Meyer[1], Daniel R Davies[1], Jeffrey L. Barr[1,2], Pasquale Manzerra[1] and Gina L. Forster[1]*

[1]*Neuroscience Group, Division of Basic Biomedical Sciences, Sanford School of Medicine, University of South Dakota, Vermillion, SD, 57069, USA.*
[2]*Department of Pharmacology, Temple University School of Medicine, Philadelphia, PA, 19140, USA*

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***Corresponding Author**
Gina L. Forster, Ph.D.
Division of Basic Biomedical Sciences
Sanford School of Medicine
University of South Dakota
414 E. Clark St.
Vermillion, SD, 57069
USA
Telephone: (605) 677 6883
Fax: (605) 677 6381
email: gforster@usd.edu

Abbreviations

ACC	anterior cingulate cortex
BLA/LA	basolateral/lateral amydala
CeA	central nucleus of the amygdala
Cg	cingulate cortex
Cx	cortex
DG	dentate gyrus
EPM	elevated plus maze
GAD	Generalized anxiety disorder
IL	infralimbic cortex
MeA	medial amygdala
mPFC	medial prefrontal cortex
mTBI	mild traumatic brain injury
PBS	phosphate-buffered saline
PrL	prelimbic cortex
PTSD	posttraumatic stress disorder
RT	room temperature
SNK	Student-Newman-Keuls

Abstract

Recent reports suggest that experiencing a mild closed head trauma or mild traumatic brain injury (mTBI) is associated with a greater incidence of anxiety disorders. Dysfunction of limbic structures, such as the medial prefrontal cortex, amygdala and hippocampus, is associated with the symptoms of anxiety disorders. Therefore, the goal of the current studies was to characterize the consequences of closed mTBI on these limbic structures and associated fear and anxiety-related behaviors. A weight-drop procedure was employed to induce mTBI in male rats. Rats were transcardically perfused 4 or 9 days following exposure to mTBI or control procedures, and neuronal number, brain region area, and the number of apoptotic cells in each region were determined. In separate groups of rats, the effects of mTBI on anxiety-like behaviors, motor function, nociception, and acquisition, retention and extinction of contextual fear were also assessed. Findings suggest that mTBI was associated with significant neuronal cell loss in the CA1 region of the dorsal hippocampus and increased cell number in subregions of the amygdala, both of which appear to be related to alterations to apoptosis in these regions following mTBI. Furthermore, mTBI increased expression of anxiety-like behaviors and conditioned fear, with no effect on motor performance or nociception. Overall, a single impact to the skull to mimic mTBI in rats produces discrete alterations to neuronal numbers within the limbic system and specific emotional deficits, providing a potential neurobiological link between mTBI and anxiety disorders.

Key Words

Mild traumatic brain injury; Concussive injury; Apoptosis; Hippocampus; Amygdala; Anxiety; Posttraumatic stress disorder.

Introduction

Mild traumatic brain injury (mTBI) is characterized by a non-penetrating direct or indirect blow to the head, accompanied by loss of consciousness for less than 30 min and/or alterations to mental state (Bay and Liberzon, 2009; Bonne et al., 2003; Gaylord et al., 2008; Hoge et al., 2008; Moore et al., 2006). Up to 1.5 million individuals in the US suffer from a mTBI annually, and these injuries most often occur in young males (Moore et al., 2006). While most individuals fully recover from mTBI within 12 weeks, estimates suggest that 7-30% (even up to 60% in some reports) of individuals suffer from a post-concussive syndrome that comprises physical, cognitive and emotional symptoms resulting in impairment for months to years (Bay and Liberzon, 2009; Belanger et al., 2007; Kennedy et al., 2007; Lewine et al., 2007; Ptito et al., 2007). Specifically, 23% of individuals that sustain mild brain injury suffer from an anxiety disorder as a result of the injury, most often generalized anxiety disorder (GAD) or posttraumatic stress disorder (PTSD) (as reviewed by Moore et al., 2006). Furthermore, a recent survey of US Army infantry soldiers revealed close to 15% suffered a mTBI, and of those, 44% also met the diagnosis criteria for PTSD (Hoge, et al., 2008). Distinguishing symptoms of post-concussive syndrome from those of anxiety disorders such as GAD or PTSD can be difficult, given many overlapping non-specific symptoms such as irritability, insomnia and amnesia (Gaylord et al., 2008; Moore et al., 2006). However, the potential for comorbidity of post-concussive syndrome with anxiety disorders has recently been discussed (e.g. Bryant, 2008; Gaylord et al., 2008; Hoge et al., 2008; Kennedy et al., 2007; Moore et al., 2006).

Brain regions susceptible to shearing and bruising during a mTBI incident involve the frontal pole and parts of the limbic system including the amygdala and hippocampus, which are also important regions for processing emotion and regulating behavioral and physiological

responses to stressors (Bryant, 2008; Kennedy et al., 2007). The amygdala is a key structure involved in emotional processing and activating stress responses, and plays an important role in acquisition and expression of fear conditioning (Goosens and Maren, 2001; Herman et al., 2005). Functionally, a large number of imaging studies with various PTSD populations show hyperactivity of the amygdala in response to negatively-valenced emotive stimuli when compared to non-PTSD controls (e.g. Armony et al., 2005; Liberzon et al 1999; Milad et al., 2006; Rauch et al., 2000, 2006; Shin et al., 2004; 2005). In contrast to the amygdala, many functional imaging studies with PTSD subjects show hypoactivity within the anterior cingulate cortex (ACC; Brodman areas 24, 25, 32 and 33) during emotive imagery or when subjects are required to suppress trauma-related information in order to produce a correct response (e.g., Bremner et al., 2004; Hopper et al., 2007; Milad et al., 2006; Shin et al, 2001, 2005). Likewise, the hippocampal activation during the presentation of negatively-valanced words is attenuated in PTSD populations (Bremner et al., 2003). Interestingly, the ACC (or the equivalent medial prefrontal cortex [mPFC] of rats; Milad et al., 2006) and hippocampus are thought to play a role in reducing stress responsiveness (Herman et al., 2005), and the mPFC in particular inhibits amygdala activity and output (Milad et al, 2006; Quirk et al., 2003). During heightened anxiety states, there appears to be inadequate top-down inhibition of the amygdala by the ACC or mPFC (Milad et al., 2006; Rauch et al., 2006). Therefore, it is conceivable that alterations to ACC (mPFC), hippocampus and amygdala function by mTBI could result in symptoms that resemble PTSD or other anxiety disorders.

To directly test the above hypothesis, a suitable animal model is required. While there are many experimental models of TBI such as fluid percussion or controlled cortical impact, most require section of the skull to be removed and direct trauma to the brain tissue, producing

moderate to severe TBI (Morales et al., 2005). A modified weight drop method for adult rats and mice allows for a mild closed head injury that more closely resembles a human concussion injury. Specifically, this method does not lead to gross morphological damage to the rat brain (Henninger et al., 2005, 2007; Tang et al., 1997b), similar to what is often seen with human post-concussive syndrome (Anderson et al., 1996; Belanger et al., 2007; Wallesch et al., 2001). However, this injury results in discrete cell loss in the rat hippocampus as measured 9-14 days after the injury, accompanied by deficits in spatial learning and memory (Fujiki et al., 2008; Henninger et al., 2005; Tang et al., 1997a,b). Furthermore, concussive mTBI in mice results in increased anxiety-like behaviors as measured 30 days following the injury (Baratz et al., 2010). Together, these findings suggest that mTBI can result in discrete damage to the limbic system to have functional behavioral consequences. However, cell loss in other limbic regions that could mediate anxiety-like states have not been examined and related to anxiety or fear behaviors following mTBI in an animal model. Using an equivalent model of mTBI in young adult male rats, we tested whether this injury resulted in alterations to neuronal number and cell death in other regions of the limbic system (mPFC and subregions of the amygdala) in addition to the hippocampus, and hypothesized that disruption in the limbic system would result in increased fear and anxiety following mTBI.

Materials and methods

Animals

The experiments conducted were approved by the Institutional Animal Care and Use Committee of South Dakota and the US Army Medical Research and Materiel Command Animal Care and

Use Office, and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

Sixty-eight young adult male Sprague-Dawley rats (8-12 weeks old, Animal Resource Center, The University of South Dakota, Vermillion, South Dakota) were housed in pairs at a constant room temperature (22°C, 60% relative humidity) and with a reverse 12 h light: 12 h dark cycle (lights off at 10:00 am). Food and water were available *ad libitum.* Rats within each pair were randomly assigned to a weight-drop (mTBI) or sham surgery (control) group. All behavioral testing was conducted at least 1 hour after the onset of the dark phase (active phase) of the light cycle in dark rooms illuminated by red lighting.

Surgery to Induce mTBI

The mild weight-drop model was used in these experiments, as this closed-head injury with impact to the skull is a minimally invasive method that does not require craniotomy, mimicking concussive head injury (Henninger et al., 2005; Weber 2007). The laboratory-made weight drop apparatus used in these experiments was based on that used by Henninger et al. (2005, 2007) and Fujiki et al. (2008) to induce mTBI in adult rats. The following parameters were calibrated to induce a mild head injury in the absence of skull fractures, subdural, subarachnoid, intraventricular or brain hemorrhages, lesions or contusions (Henninger et al., 2005; Tang et al., 1997b) as determined by pilot studies examining the brain and skull at 1 hr, 24 hrs, 4 days and 9 days following injury. The apparatus had a Plexiglas® tube (inner diameter = 11 mm, length = 100 cm) and holes were drilled at 2 cm intervals to minimize friction. A 175 g cylindrical brass weight (10 mm diameter) was allowed to freely drop through the tube onto a cylindrical

polyacetal transducer rod (diameter = 10 mm, weight = 32.6 g, length = 15.75 cm). The weight was dropped from a distance of 42 cm by pressing the release on the solenoid, ensuring consistency between drops.

Rats were induced with 4% isoflurane in 3.0 L/min O_2, and the isoflurane was reduced to 3% throughout the rest of the surgical process. After induction, rectal temperature was monitored and maintained at 37°±0.5°C by a feedback system heating pad (Harvard Apparatus, Holliston, MA). Pulse, breath rate, and oxygen saturation were monitored using a foot-clip oximeter device (Stoelting, Wood Dale, IL). The skull was exposed using a midline incision and the rat was moved under the weight drop apparatus in the prone position. The transducer rod was positioned on midline posterior to bregma with the head secured between two Plexiglas® blocks and held manually in the back to prevent any movement. Adjustments were then made to ensure the head was positioned level was at a 90 degree angle to the transducer. Once correctly positioned, the weight was released, and the transducer rod held immediately following contact to avoid rebound injuries. The wound was then closed using wound clips and the animal was removed from isoflurane. Rats were given an intramuscular injection of the analgesic ketoprofen (5 mg/kg) and monitored for a righting reflex. Control rats underwent the same surgical procedures for the same length of time under isoflurane, in the absence of the weight drop.

Histology

All animals used for histological analysis were transcardially perfused either four or nine days (n = 7 per group at each time point) following mTBI or control surgery. The nine day time point was chosen to be consistent with the other previous reports of mTBI in adult rats that assessed cell number in the dorsal hippocampus (Henninger et al., 2005; Fujiki et al., 2008), whereas the

four day time point was chosen to represent an intermediate time point to assess the progression of changes in cell number.

Rats were anesthetized using sodium pentobarbital (100 mg/kg, ip.) then perfused with phosphate-buffered saline (PBS, pH 7.4; room temperature [RT]), followed by 4% paraformaldehyde (pH 7.4, 4°C). Following perfusion, brains were post-fixed for 1 h in 4% paraformaldehyde (4°C) and then store in 25% sucrose for 2 days at 4°C. Brains were then embedded in a gelatin embedding solution (10% gelatin, 8% sucrose) and were stored in 25% sucrose for at least 2 days at 4°C. Brains were sectioned using a sliding microtome in the coronal plane, anterior to posterior at 60 μm with every third section saved. Sections were stored in cryoprotectant at -20° C until processing.

Sections were processed for either cell counting or immunohistochemistry to detect apoptotic cells. Sections used for cell counting were mounted on gelatin-coated slides, dehydrated, stained with 0.5% cresyl violet (Henninger et al., 2005) and coverslipped with cytoseal (Richard-Allan Scientific, Kalamazoo, MI). Immunohistochemistry sections were processed by co-labeling for NeuN (a neuron specific nuclear protein as a marker of mature neurons; Mullen et al., 1992) and TUNEL for apoptotic cells (Tashlkov et al., 2007). Sections were rinsed in 0.1 M PBS, incubated in mouse anti-NeuN (1:750, Chemicon International Inc, Temecula, CA) at 4 ^{o}C for 20 h, rinsed 3X for 5 min in 0.1 M PBS and incubated in Cy2-conjugated goat anti-mouse secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA) for 2 h at RT. Sections were mounted on gelatin-coated slides, air-dried, and then TUNEL staining was performed using an Apoptag red detection kit (Millipore, Temecula, CA; Barr et al., 2010). Slides were then dehydrated, and coverslipped with cytoseal (Richard-Allan Scientific).

Sections were examined using a Zeiss Axioskop 2 mot-plus microscope and all measures and counts were obtained blind to treatment. Limbic areas of interest included the sub-regions of the dorsal and ventral hippocampus, mPFC, amygdala, and the cortex below the site of injury (comprising the retrosplenial, M1, M2 and S1 trunk region cortices) as defined by Paxinos and Watson (1998). The volume of each brain region of interest was measured from cresyl violet stained sections at 2.5x magnification by the Cavaleri method (Slomianka and West, 2005), using points spaced at 10 mm, 12.5 mm, or 20 mm intervals along the x and y axis. The area associated with each point was 100 mm^2, 156.25 mm^2, or 400 mm^2 respectively. The Optical Dissector method (Slomianka and West, 2005; West, 1993) was used to estimate how many neurons were present per region in cresyl violet stained sections at 20x magnification, using a 3 mm x 3 mm counting frame applied 5 or 10 times per region depending on the region size. Since the level TUNEL-labeled cells are low in number (Barr et al., 2010), the total number of positive cells were counted from NeuN-TUNEL stained sections within a counting frame that outline the region of interest on each section (defined by NeuN staining) as according to the method of Barr et al. (2010). Zeiss AxioVision software v4.5 was used to draw the counting frame and provided the area (mm) sampled. The number of TUNEL-positive cells per area sampled was calculated for each region of interest.

Contextual Fear Conditioning

Rats (n = 10 per group) were tested for contextual fear conditioning beginning 8 days after the mTBI or sham surgery procedure. The procedures follow those of Hunsaker and Kesner (2008) who demonstrated that the dorsal CA1 was important for the retrieval of contextual fear conditioning, given that mTBI resulted in loss of dorsal CA1 neurons (see Figure 2B). On the

first day of testing (encoding), rats were placed in the foot shock chamber with an overhead camera (30cm x 30cm; Noldus Information Technology, Wageningen, The Netherlands) within a sound-attenuating chamber (Med-Associates, St. Albans, VT) for 2 min prior to delivery of 10 electric foot shocks (2 s duration, 0.75mA) separated by 74 s. Shocks were delivered through the grid floor and were controlled by Ethovision 3.1. (Noldus Technologies). Video footage was recorded by Mediacruise (Canopus Co., Ltd., Nevada City, CA). Following the 10 foot shocks, animals remained in the chamber for an additional 2 min. Twenty-four hours later, rats were tested for contextual conditioning by being placed in the same chamber without shocks administered for a total of 8 min. Extinction of the conditioned fear response was examined on the third and fourth days, where the rat was again placed in the context for 8 min. Freezing behavior from video footage during the foot shock on encoding day, conditioning test and extinction days was hand-scored by a well-trained observer blind to treatment using Ethovision 3.1. Freezing was defined as total immobility of the animal with the exception of the very limited movement necessary for respiration (Forster et al., 2006).

Elevated Plus Maze Testing

A separate group of rats (n = 10 per group) were tested for unconditioned anxiety-like behaviors 6 days following mTBI or sham surgery using the elevated plus maze (EPM; Vuong et al., 2010). The maze consisted of two intersecting runways (12 cm wide x 100 cm long) which were perpendicular from each other. Two of the runways had no walls while the others had high walls (40 cm high), with the maze elevated 1 m from the floor (Noldus Information Technology, Wageningen, The Netherlands). The test started with a rat being placed in the center of the maze facing a closed arm, and the rat was allowed to freely explore for 5 min. Latency to enter an

open arm, cumulative time spent in each arm, and total distance moved within the maze were automatically scored by Ethovision XT v5.1 (Noldus Technologies).

Locomotion

Rats that had been used for EPM testing (n = 10 per group) were tested for locomotor behavior in the foot shock chambers 7 days following mTBI or sham surgeries. This was conducted to ensure that any differences observed in the conditioned foot shock experiment were not due to differences in activity within the chambers between mTBI and control groups. Animals were placed in the same chambers used for the foot shock experiment as described above and allowed to freely move around the chamber for 30 min. Total distance moved was scored automatically (Ethovision 3.1, Noldus Technologies).

Rotarod Testing

Rats that had been used for EPM and locomotion testing (n = 10 per group) were trained and tested on the rotarod for motor strength and coordination starting day 8 post-surgery. This test was used to ensure that the weight drop procedure did not produce gross motor deficits that would suggest an injury greater than mTBI (Henninger et al., 2005). The rotarod consisted of a 9.5 cm diameter barrel positioned 23 cm above a transducer platform (Stoelting, Wood Dale, IL). Animals were given five training trials of 3 min for each of two training days, allowing for at least 5 min of rest between each trial. Each training trial was started with a barrel speed of 5 rpm which accelerated to the final speed of 15 rpm over the first 90 sec. On the test day, rats were placed on the rotarod with the same rpm and acceleration rate as for the training days (modified from Malherbe et al., 2009), with the test length remaining congruent at 2 min. Rats were

allowed three tests, and latency to fall onto the transducer platform was automatically recorded by software (IITC INC./Life Science, Woodland Hills, CA). The average latency over the three trials was calculated for analysis.

Tail-withdrawal Test

Finally at 12 days post-surgery, rats that had been exposed to EPM, locomotion and rotarod testing (n = 10 per group) went through the tail-withdrawal test as according to Lukkes et al. (2009). This was conducted to ensure that any differences in foot shock reactivity or conditioning could not be attributed to differences in nociception between mTBI and control groups (Lukkes et al., 2009). Rats were acclimated to the restraining tube (20.3 x 8.9 x 5.7cm; Braintree Scientific, Inc, Braintree, MA) for 5 min per day on two consecutive days previous to testing. On the test day, the distal 10 cm of the tail was immersed in 50±0.5 °C water. The temperature of the water was maintained using a thermostat controlled water bath. The latency to withdrawal the tail from the water was recorded by a second experimenter blind to treatment using a stopwatch (0.01 s time resolution). Three trials were performed with 30 s between each trial, with the average latency over the three trials calculated.

Data Analysis

Initially, all data was subjected to the Grubbs outlier test (Feng et al., 2009), which resulted in the removal of 22 statistical outlier data points from the 1092 total data points from the histology experiments, and 2 statistical outlier data points from the 200 total data points from the behavioral experiments ($P < 0.05$). Following this, one-way ANOVA were used to analyze all cell counts and behavioral data. The exceptions to this were the physiological parameters

recorded during surgery and the fear conditioning data, which were analyzed using separate two-way ANOVA with time as a repeated measure, followed by Student-Newman-Keuls (SNK) *post-hoc* test for between-subject pairwise comparisons or Holm-Sidak *post-hoc* test for multiple pairwise comparisons over time. The significance level for all analyses was set at $P < 0.05$, and all analyses were performed using Sigma Stat v3.5.

Results

Effects of mTBI on Physiological Parameters

Control (21.67 +/- 0.73 min) and mTBI (21.61 +/- 0.94 min) rats spent similar amount of time under isoflurane during the surgical period ($F_{(1,60)} = 0.003$, $P = 0.957$). Physiological parameters were recorded following the mTBI or sham procedures during the surgery and over the subsequent recovery period between removal of isoflurane and return of righting reflex, with few differences between control and mTBI rats observed (Table 1). For oxygen saturation, there was a significant effect of time ($F_{(5,237)} = 12.380$, $P < 0.001$) but no significant effect of mTBI treatment ($F_{(1,50)} = 0.0484$, $P = 0.490$) nor a significant interaction between treatment and time ($F_{(5, 237)} = 1.751$, $P = 0.124$). Oxygen saturation significantly decreased over time for both control rats (4-8 min post-sham procedures; Holm-Sidak $P < 0.05$) and mTBI rats (6-8 min post-mTBI; Holm-Sidak $P < 0.05$) (Table 1). Heart rate remained unaffected, with no significant effect of treatment ($F_{(1,50)} = 0.001$, $P = 0.988$), time ($F_{(5,237)} = 1.825$, $P = 0.109$) nor an interaction between treatment and time ($F_{(5,237)} = 1.374$, $P = 0.255$). However, there was a significant effect of time ($F_{(5, 237)} = 8.423$, $P < 0.001$) and a significant interaction between time and mTBI treatment ($F_{(5,237)} = 2.582$, $P = 0.027$) on breath rate. Breath rate was significantly

higher at 6 and 8 min post-mTBI, and 8 min post-sham procedures (Holm-Sidak $P < 0.05$) as compared to pre-procedure rates (Table 1). There was also a significant difference between control and mTBI in breath rate at 6 min post- procedure (SNK $P < 0.05$; Table 1). Finally, mTBI rats (5.27 +/- 0.30 min) took significantly longer to exhibit a righting reflex following the removal of isoflurane as compared to control rats (4.31 +/- 0.24 min) ($F_{(1,60)} = 6.195$, $P = 0.016$).

Effects of mTBI on the Area of Specific Limbic Regions

Figure 1 shows low-magnification examples of cresyl violet stained sections from control and mTBI rats, illustrating a lack of gross morphological damage following mTBI. The area (mm^2) of most limbic brain regions studied was not affected by mTBI, with the exception of the basolateral/lateral amygdala (BLA/LA) and the medial amygdala (MeA) (Table 2). The area of the BLA/LA was not affected by mTBI at 4 days post-injury ($F_{(1,9)} = 0.177$, $P = 0.689$), but mTBI did result in a significant increase in the area of the BLA/LA 9 days following injury ($F_{(1,9)} = 7.382$, $P = 0.026$; Table 2). Likewise, the area of the MeA was not affected by mTBI 4 days post injury ($F_{(1,9)} = 1.267$, $P = 0.293$), but there was a significant increase in the area of this region at 9 days post injury ($F_{(1,9)} = 7.812$, $P = 0.023$; Table 2).

Effect of mTBI on the Total Number of Neurons in Specific Limbic Regions

Overall, mTBI had little effect on the total number of neurons in the limbic brain regions measured (Table 3), with the exception of the dorsal hippocampus and the amygdala (Figures 2-3). Specifically, the BLA/LA of mTBI rats exhibited increased neuronal number 9 days following injury ($F_{(1,9)} = 6.201$, $P = 0.038$; Figure 2B) that was not seen at 4 days post-injury ($F_{(1,8)}=0.008$, $P = 0.931$; Figure 2A). Likewise, there was no significant effect of mTBI at 4

days post-injury in the MeA ($F_{(1,9)} = 2.012$, P = 0.194; Figure 2C), with a significant increase in neuronal number within this same region at 9 days after mTBI ($F_{(1,9)} = 9.348$, P = 0.016; Figure 2D). In contrast, mTBI had no effect on the total number of neurons in the CeA at 4 ($F_{(1,9)} = 0.001$, P = 0.970; Figure 2E) or at 9 days after injury ($F_{(1,10)} = 0.173$, P = 0.686; Figure 2F).

Opposite to the amygdala, mTBI led to a decrease in the total number of neurons in the CA1 region of the dorsal hippocampus 9 days after injury ($F_{(1,11)} = 5.242$, P = 0.043; Figure 3B), but not at 4 days ($F_{(1,11)} = 0.104$, P=0.753; Figure 3A). In contrast, mTBI had no effect on total neuronal number in the dorsal CA3 region at 4 days ($F_{(1,11)} = 0.062$, P = 0.807; Figure 3C) or at 9 days post-injury ($F_{(1,12)} = 0.002$, P = 0.964; Figure 3D), nor on the total number of neurons in the dorsal DG at 4 ($F_{(1,10)} = 4.329$, P = 0.064; Figure 3E) or 9 days post-injury ($F_{(1,12)} = 0.307$, P = 0.590; Figure 3F).

Effect of mTBI on Apoptosis in Specific Limbic Regions

Figure 4 shows low- and high-magnification examples of TUNEL-positive staining of sections from control and mTBI rats. TUNEL-positive cells were often co-localized with NueN-positive cells (e.g. Figure $4A_3$ and $4D_3$) indicative of apoptotic neurons. However, many TUNEL-positive cells were not co-localized with NueN (e.g. Figures $4D_1$ $4B_3$). These can either represent apoptotic glia or neurons in the final stages of apoptosis where DNA breakdown prevents the synthesis of NeuN (Biebl et al. 2000; Barr at al., 2010). Since these possibilities cannot be distinguished, all TUNEl cells were counted, with the caveat that these might not all represent neuronal cell types.

The level of apoptosis did not differ between control and mTBI groups in a number of the limbic brain regions measured (Table 4), with the exception of the amygdala and the dorsal

hippocampus (Figures 3-4). Specifically, the density of apoptotic cells in two subregions of the amygdala (Figure 3) was significantly decreased following mTBI. The BLA/LA had decreased density of apoptotic cells following mTBI at both 4 days ($F_{(1,10)} = 5.712$, $P = 0.041$; Figure 3A) and 9 days ($F_{(1,11)} = 7.193$, $P = 0.021$; Figure 3B). Similarly, there was a decreased density of apoptotic cells in the MeA at both 4 days ($F_{(1,10)} = 41.409$, $P < 0.001$; Figure 3C) and 9 days ($F_{(1,11)} = 5.227$, $P = 0.043$; Figure 3D) post-mTBI. There was no significant effect of mTBI on apoptosis in the CeA at 4 days ($F_{(1,10)} = 0.0452$, $P = 0.836$; Figure 3E) or at 9 days ($F_{(1,11)} = 0.104$, $P = 0.754$; Figure 3F).

Apoptosis was increased in two subregions of the dorsal hippocampus following mTBI (Figure 4). There was a significant increase in density of apoptotic cells in the dorsal CA1 at 4 days ($F_{(1,11)} = 5.305$, $P = 0.042$; Figure 4A) post-mTBI, but not at 9 days ($F_{(1,11)} = 2.903$, $P = 0.116$; Figure 4B). However, there was no effect of mTBI on apoptosis in the dorsal CA3 as measured at 4 days ($F_{(1,11)} = 1.135$, $P = 0.310$; Figure 4C), or at 9 days ($F_{(1,11)} = 0.341$, $P = 0.571$; Figure 4D). Similar to the dorsal CA1, mTBI increased the density of apoptoic cells in the dorsal DG at 4 days ($F_{(1,11)} = 6.165$, $P = 0.030$; Figure 4E), but not 9 days ($F_{(1,11)} = 0.193$, $P = 0.669$; Figure 4F).

Effects of mTBI on Contextual Fear Conditioning and Nociception

Overall, mTBI increased the acquisition of contextual fear conditioning without affecting extinction of this response, or altering nociception (Figure 5). During conditioning, there was no significant effect of mTBI on the percent of time spent freezing ($F_{(1,17)} = 1.195$, $P = 0.290$; Figure 5A). However, for the conditioning test and extinction days (Figure 5B), there was a significant effect of mTBI ($F_{(1,18)} = 5.597$, $P = 0.029$), and a significant effect of test day ($F_{(2,31)}$

= 47.495, P < 0.001). *Post hoc* analysis revealed mTBI rats exhibited significantly increased time spent freezing on the conditioning test day as compared to control rats (SNK P = 0.029; Figure 5B). Furthermore, both mTBI and control rats spent significantly less time freezing on extinction day 1 (Holm-Sidak P < 0.001) and extinction day 2 (Holm-Sidak P < 0.001) when compared to the conditioning test day, but there was no significant difference in the degree of freezing behavior between mTBI and control groups on extinction days (SNK P > 0.05; Figure 5B). When nociception was testing using the tail withdrawal test, there was no significant difference between control and mTBI groups ($F_{(1,18)}$ = 1.292, P = 0.271; Figure 5C).

Effects of mTBI on Anxiety-like Behavior

Testing on the EPM revealed that mTBI also resulted in increased unconditioned anxiety-like behaviors (Figure 6). Specifically, mTBI resulted in significantly higher latency to enter an open arm ($F_{(1,11)}$ = 10.55, P = 0.009; Figure 6A), and reduced time spent in the open arms of the maze ($F_{(1,18)}$ = 9.431, P = 0.007; Figure 6B). These differences were not a result of decreased mobility following mTBI, since the distance moved in the entire maze was not significantly different between the two groups ($F_{(1,18)}$ = 2.509, P = 0.130; Figure 6C).

Effects of mTBI on Locomotion, Strength and Coordination

The mTBI injury used in the current study did not lead to motoric deficits (Figure 7). There was no significant effect of mTBI on locomotor behavior ($F_{(1,18)}$ = 3.413, P = 0.082; Figure 7A). Furthermore, strength and coordination as tested by time spent on the rotarod, was not affected by mTBI ($F_{(1,18)}$ = 1.341, P = 0.264; Figure 7B).

Discussion

Similar to the findings of Henninger et al. (2005), mTBI in an adult rat model resulted in a significantly longer return of the righting reflex following the weight drop procedure, which was not a function of increased time under anesthesia as compared to control rats. However, the mTBI procedure did not result in gross motoric deficits when tested days after the injury. The injury did result in discrete changes in neuronal numbers in the dorsal CA1, BLA/LA, and MeA. With the limitations of TUNEL-positive cell sampling in mind (e.g. inability to definitively identify cell type, and the use of a semi-quantitative method due to low number of positive cells), the current study demonstrates that changes in neuronal numbers in the hippocampus and amygdala were accompanied by alterations to apoptosis in these same regions. Furthermore, this mTBI procedure was associated with greater unconditioned anxiety and contextual fear conditioning. The changes in neuronal numbers, apoptosis and related behaviors did not appear to be due to observable hypoxia or ischemia during the mTBI event, particularly since the injury did not cause any bleeding within or around the brain, and saturated O_2 levels did not differ between sham and mTBI groups. Therefore, the mild impact to the skull appears to result in discrete changes to the limbic system and emotive behaviors that are apparent days following injury without obvious gross morphological damage to the brain or motoric deficits.

Previous (e.g.: Fujiki et al., 2008; Henninger et al., 2005; Tang et al., 1997b) and current findings suggest that the dorsal hippocampus is susceptible to neuronal loss following mild head injury. Specifically, mTBI injury of adult rats or mice often results in neuronal loss in the CA1 and CA3 regions of the dorsal hippocampus, as measured 9-14 days post-injury (Fujiki et al., 2008; Henninger et al., 2005; Tang et al., 1997b). The current study differs from some previous reports (e.g. Henninger et al., 2005; Tang et al., 1997b) but is similar to others (e.g., Fujiki et al.,

2008) by observing significant cell loss in the dorsal CA1 region only, suggestive of a milder injury. However, the current study adds to previous findings by showing that up to 9 days is required to observe neuronal loss in the dorsal CA1 after mTBI, since this cell loss is not observable 4 days post-injury. Delayed neuronal loss in the dorsal CA1 may be a result of injury-induced onset of apoptotic mechanisms, since increased density of apopotic cells in the CA1 region was observed 4 days post-injury. In support of this, mTBI in a mouse model leads to increased expression of proapoptotic proteins such p53 in whole brain homogenates and caspase 3, Bax and apoptosis-inducing factor in the hippocampus, 72 hours following injury (Tweedie et al., 2007; Tashlykov et al., 2009). Overall, the current findings complement others that suggest that the CA1 region of the dorsal hippocampus is often more susceptible to neuronal cell loss following perturbation (Schmidt-Kastner and Freund, 1991; Kreisman et al, 2000; Fujiki et al., 2008). Kreisman et al. (2000) suggest that increased susceptibility of CA1 neurons is a result of increased excitotoxic events in this region immediately after an ischemic or hypoxic event. While enhancement of excitatory synaptic activity and hyperactivity of CA1 neurons and regional CA1 increases in glucose metabolism have been observed immediately following moderate fluid percussive brain injury (Yoshino et al., 1992; Akasu et al., 2002; Ooba et al., 2009), this has not been studied directly in models of closed-head concussive injury (i.e. mTBI) and thus the mechanisms by which neurons in the CA1 region are susceptible to mTBI require further examination.

The dorsal DG showed also increased apoptosis 4 days post-injury in the current study, but this did not manifest as decreased neuronal number at 4 or 9 days following mTBI. Other reports also suggest increased apoptosis in this region 72 hours following mTBI (Tashlykov et al., 2007, 2009). However, the lack of concordance between heightened apoptosis and ultimate

neuronal number may be a result of the DG being a locus of adult neurogenesis (Ehninger and Kempermann, 2008), so that increased injury-induced apoptosis may be compensated for by increased neurogenesis in this region. Cell proliferation in the DG is rapidly induced following TBI induced by both focal (e.g. fluid percussion or cortical impact) or diffuse (e.g. impact-acceleration) injuries, and peaks between 3 and 7 days post injury (Dash et al., 2001; Emery et al., 2005; Sun et al., 2007; Bye et al., 2011). This increased production of cells leads to greater numbers of newly integrated dentate granule neurons (Emery et al., 2005; Sun et al., 2007) despite reduced survival rates (Sun et al., 2007). While this has not been studied following closed-head injury mTBI, findings from other TBI models suggest that mTBI-induced apoptosis may be compensated for by increased neurogenesis in this region.

The current findings also demonstrate for the first time that the amygdala is also susceptible to mTBI. In particular, mTBI resulted in increased neuronal numbers in the BLA/LA and the MeA 9 days post-injury, which is likely a result of decreased apoptosis in these regions as observed 4 days post-injury. Interestingly, decreased apoptosis was also observed in the BLA/LA and MeA at 9 days post-injury, suggesting that cell numbers in this region may continue to increase beyond the 9 day observation point. The area of each region was also significantly increased by 9 days post-injury, probably a function of increased neuronal number and decreased cell death. While not an area of focus in adult mTBI imaging studies, increased amygdala volume has also been observed in a 10 year follow up of childhood TBI (Beauchamp et al., 2011). An increase in neuronal number and decreased levels of apoptosis suggest that like cortical impact and moderate/severe weight drop models, mTBI might activate cell survival molecules such as bcl-2 (Clark et al., 1997), PKB/Akt (Noshita et al., 2002, Shapira et al., 2007, Zhang et al., 2006), and IGF-1/IGF-1R (Madathil et al., 2010, Rubovitch et al., 2010) to decrease

programmed cell death in these sub regions of the amygdala. Thus, whether these markers are increased in the amygdala following mild TBI should be explored. Interestingly, suppression of cell death in the amygdala has been reported in mice exposed to environmental enrichment (Okuda et al., 2009), suggesting that such a phenomenon might occur in response to both positive and negative perturbations.

Human studies suggest that mTBI increases the incidence of anxiety disorders, particularly PTSD and GAD (e.g. Moore et al., 2006). Using the EPM, the current study showed that rats exposed to mTBI exhibit increased latency to enter open arms and reduced time in open arms, similar to the effects of mTBI in mice (Baratz et al., 2010). We also show that the increase in anxiety-like behaviors is independent of overall locomotion. The current and past EPM findings are indicative of increased unconditioned anxiety-like behaviors following mTBI, suggesting that this may be a good animal model to investigate the neurobiological mechanisms by which mTBI results in GAD in some individuals. The amygdala and the hippocampus (via the septo-hippocampal system) have both distinct and overlapping roles in the expression of anxiety states, with the hippocampus playing a greater role in negative cognitive bias characterizing GAD while the amygdala appears responsible for heightened arousal associated with GAD (McNaughton and Corr, 2004). While changes to neuronal numbers cannot be directly related to function within the same regions without further experimentation, it is feasible that the altered neuronal numbers in the sub regions of the amygdala and dorsal hippocampus observed following mTBI result in an imbalance in activity that ultimately increases unconditioned anxiety behavior. This is supported for the amygdala in particular by human imaging studies that show larger amygdala volumes in patients that suffer from GAD (Etkin et

al., 2009; Schienle et al., 2011), concordant with the larger area of amygdala sub regions following mTBI in the current study.

Contextual fear conditioning was also heightened by mTBI in the current study, independent of any effect of mTBI on nociception. Suffers of PTSD also show heightened contextual fear conditioning in both experimental and natural settings (e.g. Grillon, 2002; Grillon and Morgan, 1999), suggesting that this rat model approximates some of the symptoms of PTSD. Alterations to the hippocampus and amygdala induced by mTBI may underlie the observed enhancement in contextual fear conditioning. For example, the dorsal CA1 is implicated in the expression of contextual fear conditioning, although lesions of this region cause impairment in the acquisition of contextual fear (Hunsaker and Kesner, 2008). One possible explanation is that disruption to normal circuitry via discrete cell loss in the CA1 results in enhanced contextual fear conditioning, as seen in the current study, while full abolition of the region (Hunsaker and Kesner, 2008) decreases this behavior. Alternatively, the BLA/LA and MeA sub regions of the amygdala play an important role in the acquisition and expression of contextual fear conditioning (Fanselow and Le Doux, 1999; Goosens and Maren, 2001; Helmstetter et al., 2008), and hyperactivity of the amygdala is associated with PTSD (Armony et al., 2005; Liberzon et al 1999b; Milad et al., 2006; Rauch et al., 2000, 2006; Shin et al., 2004, 2005). Therefore, increased cell number in the amydgala induced by mTBI may result in greater activity in this region and heighted expression of contextual fear conditioning.

Human studies suggest that PTSD subjects also exhibit poorer extinction of conditioned fear responses, which is thought to be a function of frontal cortex hypofunction (Milad et al., 2006, 2008; Quirk et al., 2006). However, in the current study there was no effect of mTBI on extinction of contextual fear conditioning, matching a lack of effect of mTBI on the subregions

of the mPFC. Therefore, the current rat model does not approximate this aspect of PTSD. The majority of incidents that would result in a mTBI have a stressful component to them (Moore et al., 2006), and recent evidence suggests that brain injury and chronic stress combined have greater effects on the brain and behavior (Kwon et al., 2011). Therefore, the ability of mTBI in the rat to more closely produce PTSD-like symptoms might be enhanced by concurrent exposure to psychological stress, and this possibility requires further testing.

Conclusions

The present rat model established a direct link between mTBI and the development of increased fear conditioning and anxiety states that are similar to symptoms of GAD and PTSD. While the underlying neurobiology needs to be tested directly, the current findings suggest that mTBI-induced alterations to apoptosis and neuronal cell numbers in the dorsal hippocampus and the amygdala are associated with post-injury fear and anxiety states. Future work to establish the mechanisms by which mTBI induces discrete changes in neuronal populations, and how these manifest into emotive behavioral deficits, will significantly enhance the current understanding of the neurobiology underlying anxiety disorders following head injury.

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Figure Legends

Figure 1

Examples of cresyl violet stained sections 9 days following sham (control) or mTBI procedures, illustrating the lack of gross morphological damage following the mTBI. (**A-B**) Amygdala subregions and (**C-D**) dorsal hippocampal subregions, all at 5x magnification. BLA/LA = lateral and basolateral amygdala; MeA = medial amygdala; CeA = central nucleus of the amygdala; DG = dentate gyrus. Black scale bar = 200 μm.

Figure 2

Total number of neurons in the amygdala subregions as measured at (**A, C, E**) 4 days and (**B**, **D, F**) 9 days following mTBI or control surgery. *$P < 0.05$ compared to control group at the same time point.

Figure 3

Total number of neurons in the dorsal hippocampal subregions as measured at (**A, C, E**) 4 days and (**B**, **D, F**) 9 days following mTBI or control surgery. *$P < 0.05$ compared to control group at the same time point. DG = dentate gyrus.

Figure 4

Examples of TUNEL and NueN stained sections 4 days following sham (control) or mTBI procedures. (**A-B**) The basolateral subregion of the amygdala (outlined by white dotted line) and (**C-D**) dorsal CA1 hippocampal subregion. Arrows indicate TUNEL-positive cells. Subscript 1 panels were imaged at 10x magnification and subscript 2-3 panels were imaged at 40x

magnification. Subscript 1 and 3 panels show TUNEL-NeuN merged images while subscript 2 panels show TUNEL images.

Figure 5

Density of apoptotic cells in the amygdala subregions as measured at (**A, C, E**) 4 days and (**B**, **D, F**) 9 days following mTBI or control surgery. *P < 0.05 compared to control group at the same time point. BLA/LA = lateral and basolateral amygdala; MeA = medial amygdala; CeA = central nucleus of the amygdala.

Figure 6

Density of apoptotic cells in the dorsal hippocampus subregions as measured at (**A, C, E**) 4 days and (**B**, **D, F**) 9 days following mTBI or control surgery. *P < 0.05 compared to control group at the same time point.

Figure 7

(**A**) Level of freezing behavior in response to foot shock 8 days following mTBI or control surgery. (**B**) Time spent freezing in the context associated with foot shock on days 9-11 following mTBI or control surgery. (**C**) Latency to withdrawal tail in a thermal nocieption test 12 days following mTBI or control surgery. *P < 0.05 compared to control group on the same test day. $^{\#}$P<0.05 compared to conditioning test day within the same treatment group.

Figure 8

(**A**) Latency to enter open arms, (**B**) time spent in open arms and (**C**) total distance moved within the entire elevated plus maze, 6 days following mTBI or control surgery. *$P < 0.05$ compared to control group.

Figure 9

(**A**) Total distance moved within the foot shock chamber over 30 minutes in the absence of foot shock, 7 days following mTBI or control surgery. (**B**) Time spent on the rotorod during the 2 minute test, 10 days following mTBI or control surgery.

Table 1: *Physiological Parameters following mTBI or Sham Procedures*

Time (min)	O_2 Saturation (%)		Heart Rate (beats/min)		Breath Rate (breaths/min)	
	Control	mTBI	Control	mTBI	Control	mTBI
-1	98.82 ± 0.08	97.71 ± 0.79	301.61 ± 7.01	306.00 ± 4.80	42.69 ± 1.58	42.49 ± 1.69
0	98.22 ± 0.31	98.09 ± 0.36	299.63 ± 6.76	301.25 ± 4.92	40.28 ± 1.38	45.44 ± 2.59
2	96.75 ± 0.98	98.65 ± 0.09	304.55 ± 10.91	298.85 ± 16.33	44.34 ± 3.67	41.49 ± 2.54
4	96.94 ± 0.88$^{\#}$	95.87 ± 0.77	297.18 ± 8.14	286.19 ± 7.56	40.55 ± 2.77	48.08 ± 6.35
6	96.00 ± 0.81$^{\#}$	94.61 ± 0.86$^{\#}$	306.66 ± 12.78	290.82 ± 5.64	46.47 ± 3.48	62.99 ± 2.97$^{*\#}$
8	95.08 ± 0.61$^{\#}$	93.66 ± 1.54$^{\#}$	300.93 ± 8.55	323.00 ± 16.32	58.32 ± 4.54$^{\#}$	55.94 ± 4.90$^{\#}$

Note that isoflurane/O_2 was removed by 6 min post-mTBI or sham procedures.

* Significantly different from control within same time period ($P < 0.05$)

$^{\#}$ Significantly different compared to -1 min ($P < 0.05$)

Table 2: Area (mm^2) of Specific Limbic Regions following mTBI

Brain Region	4 Days Post-Procedure			9 Days Post-Procedure		
	Control	mTBI	P Value	Control	Day mTBI	P Value
Medial Prefrontal Cortex						
Cg	362.72 ± 67.57	280.32 ± 33.22	0.276	350.47 ± 45.46	345.60 ± 40.16	0.937
PrL	166.75 ± 28.11	118.56 ± 4.23	0.159	44.50 ± 7.61	60.62 ± 11.69	0.271
IL	65.16 ± 14.36	50.21 ± 1.39	0.289	135.57 ± 25.82	157.30 ± 31.08	0.600
Amygdala						
CeA	30.80 ± 8.46	31.80 ± 5.67	0.967	44.25 ± 10.44	34.32 ± 8.21	0.481
BLA/LA	85.07 ± 21.63	72.86 ± 16.94	0.689	121.38 ± 4.07	232.32 ± 34.27	0.026
MeA	54.21 ± 17.20	82.72 ± 18.59	0.293	132.35 ± 9.24	234.78 ± 29.77	0.023
Cortex	1323.52 ± 300.04	1650.88 ± 149.66	0.329	1732.94 ± 125.02	1555.48 ± 54.86	0.270
Dorsal Hippocampus						
CA1	674.93 ± 114.65	640.36 ± 76.03	0.806	613.82 ± 50.30	493.50 ± 18.39	0.059
CA3	334.32 ± 52.84	329.21 ± 35.47	0.937	291.43 ± 22.25	283.36 ± 17.98	0.783
DG	238.86 ± 32.34	224.27 ± 22.32	0.717	185.33 ± 12.73	166.09 ± 10.55	0.267
Ventral Hippocampus						
CA1	100.32 ± 28.61	124.96 ± 11.91	0.450	57.29 ± 10.73	60.50 ± 9.00	0.822
CA3	142.56 ± 67.62	95.74 ± 27.89	0.478	40.46 ± 7.63	38.50 ± 5.27	0.842
DG	163.33 ± 25.80	172.73 ± 16.70	0.755	38.50 ± 6.38	60.89 ± 8.52	0.057

Cg = cingulate, PrL = prelimbic, IL = infralimbic, CeA = central nucleus, BLA/LA = basolateral and lateral, MeA = medial amygdala, DG = dentate gyrus.

Table 3: Total Number of Neurons in Specific Limbic Regions following mTBI

	4 Days Post-Procedure			9 Days Post-Procedure		
	Control	mTBI	P Value	Control	mTBI	P Value
Medial Prefrontal Cortex						
CG	92160.71 ± 19337.02	78273.66 ± 9049.29	0.530	99286.45 ± 12181.15	98137.30 ± 10002.69	0.943
PrL	30545.07 ± 1175.28	44221.40 ± 7857.68	0.281	39253.33 ± 7553.60	46815.70 ± 9037.28	0.533
IL	44221.44 ± 7857.68	30545.07 ± 1175.28	0.281	12993.42 ± 2507.67	17323.28 ± 3348.31	0.336
Cortex	466522.09 ± 127608.45	561369.60 ± 58655.45	0.469	583767.00 ± 36612.65	543781.38 ± 38842.96	0.509
Ventral Hippocampus						
CA1	36422.69 ± 13129.13	37690.43 ± 4965.53	0.930	11454.19 ± 2160.06	10717.12 ± 1699.00	0.794
CA3	55758.79 ± 28659.97	32277.61 ± 12468.29	0.413	12000.61 ± 2863.12	10081.61 ± 1814.10	0.598
DG	67507.34 ± 9429.59	72323.65 ± 9231.63	0.730	10463.84 ± 2202.31	15158.82 ± 1163.10	0.101

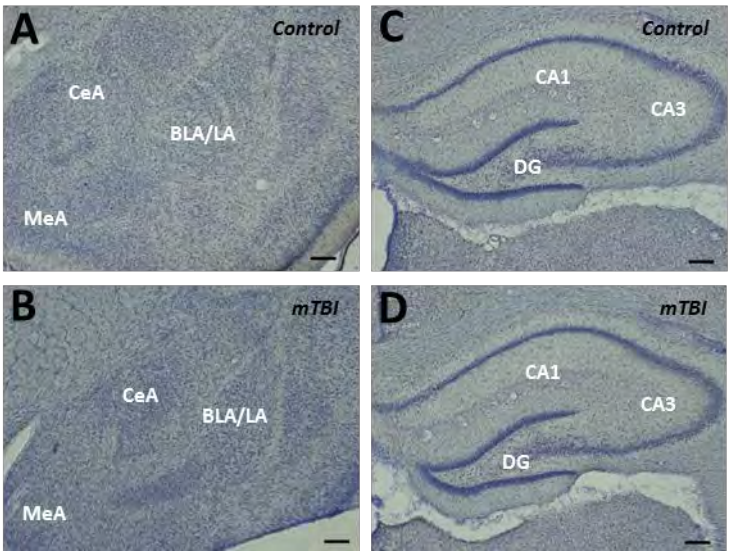
Cg = cingulate, PrL = prelimbic, IL = infralimbic, DG = dentate gyrus.

Table 4: Density of TUNEL-Positive Cells (per mm^2) in Specific Limbic Regions following mTBI

	4 Days Post-Procedure			9 Days Post-Procedure		
	Control	mTBI	P Value	Control	mTBI	P Value
Medial Prefrontal Cortex						
CG	$1.98 \pm 0.31e^{-3}$	$2.41 \pm 0.40e^{-3}$	0.419	$2.48 \pm 0.68e^{-3}$	$1.75 \pm 0.27e^{-3}$	0.368
PrL	$2.10 \pm 0.40e^{-3}$	$2.02 \pm 0.25e^{-3}$	0.866	$1.92 \pm 0.40e^{-3}$	$1.90 \pm 0.58e^{-3}$	0.982
IL	$3.55 \pm 0.54e^{-3}$	$2.38 \pm 0.21e^{-3}$	0.068	$2.53 \pm 0.41e^{-3}$	$2.10 \pm 0.37e^{-3}$	0.459
Cortex	$1.89 \pm 0.32e^{-3}$	$2.16 \pm 0.64e^{-3}$	0.803	$1.57 \pm 0.36e^{-3}$	$2.21 \pm 0.62e^{-3}$	0.394
Ventral Hippocampus						
CA1	$1.33 \pm 0.17e^{-3}$	$1.68 \pm 0.22e^{-3}$	0.271	$3.71 \pm 0.92e^{-3}$	$1.98 \pm 0.47e^{-3}$	0.137
CA3	$2.08 \pm 0.82e^{-3}$	$2.72 \pm 0.94e^{-3}$	0.647	$2.88 \pm 0.84e^{-3}$	$2.15 \pm 0.47e^{-3}$	0.490
DG	$3.57 \pm 0.69e^{-3}$	$3.02 \pm 0.30e^{-3}$	0.513	$5.23 \pm 0.68e^{-3}$	$4.36 \pm 0.83e^{-3}$	0.437

Cg = cingulate, PrL = prelimbic, IL = infralimbic, DG = dentate gyrus.

Meyer et al. Figure 1



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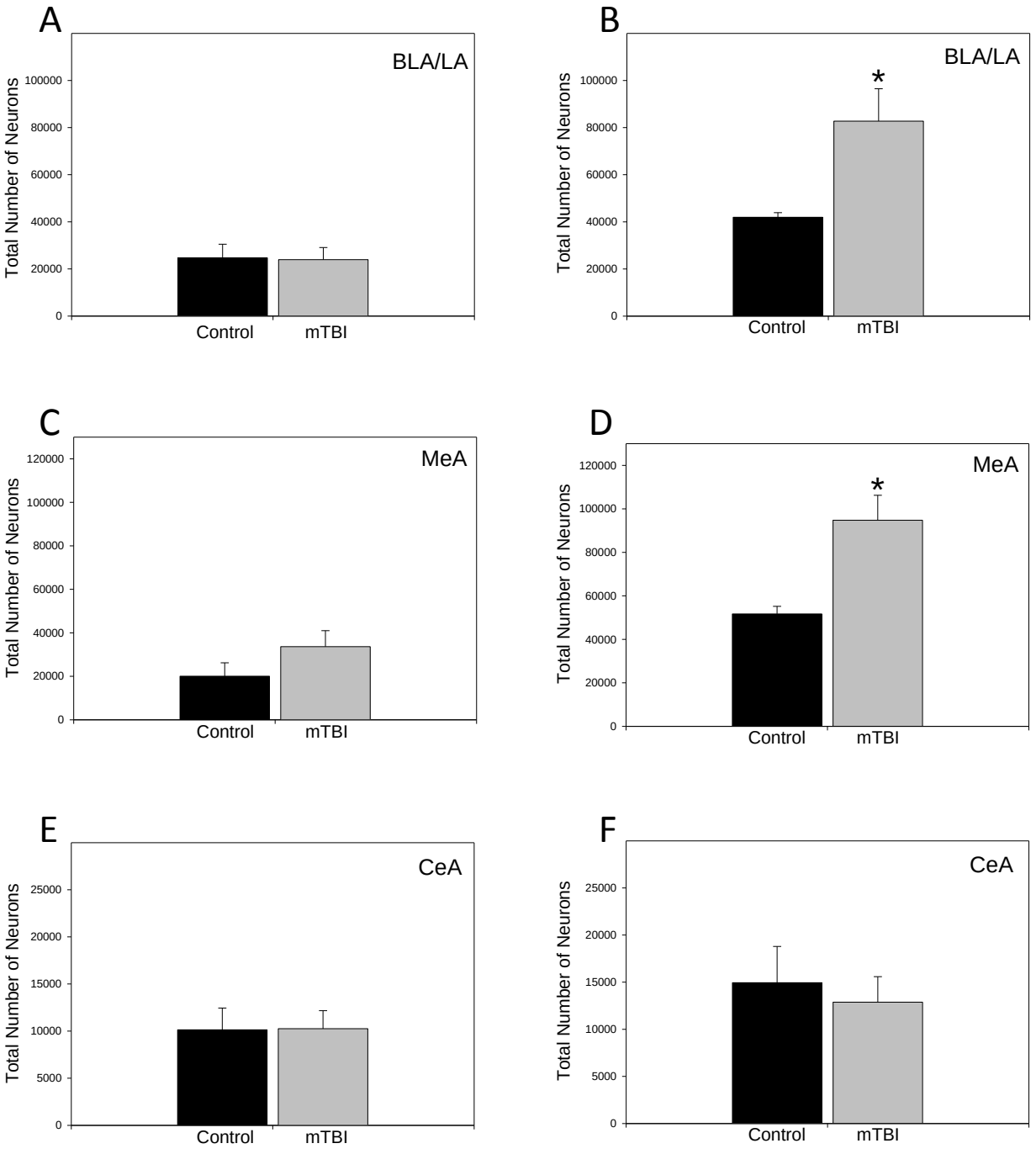

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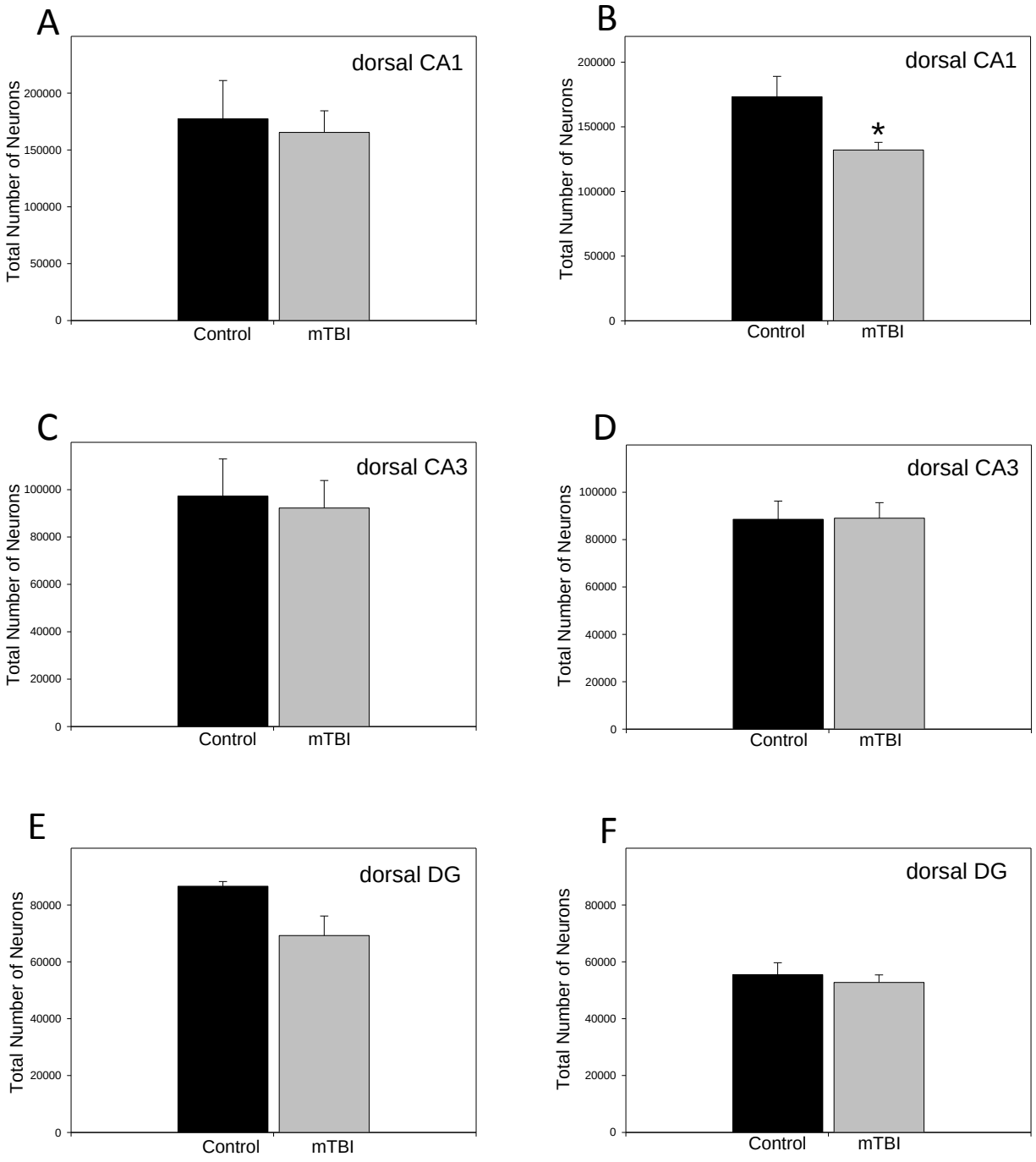

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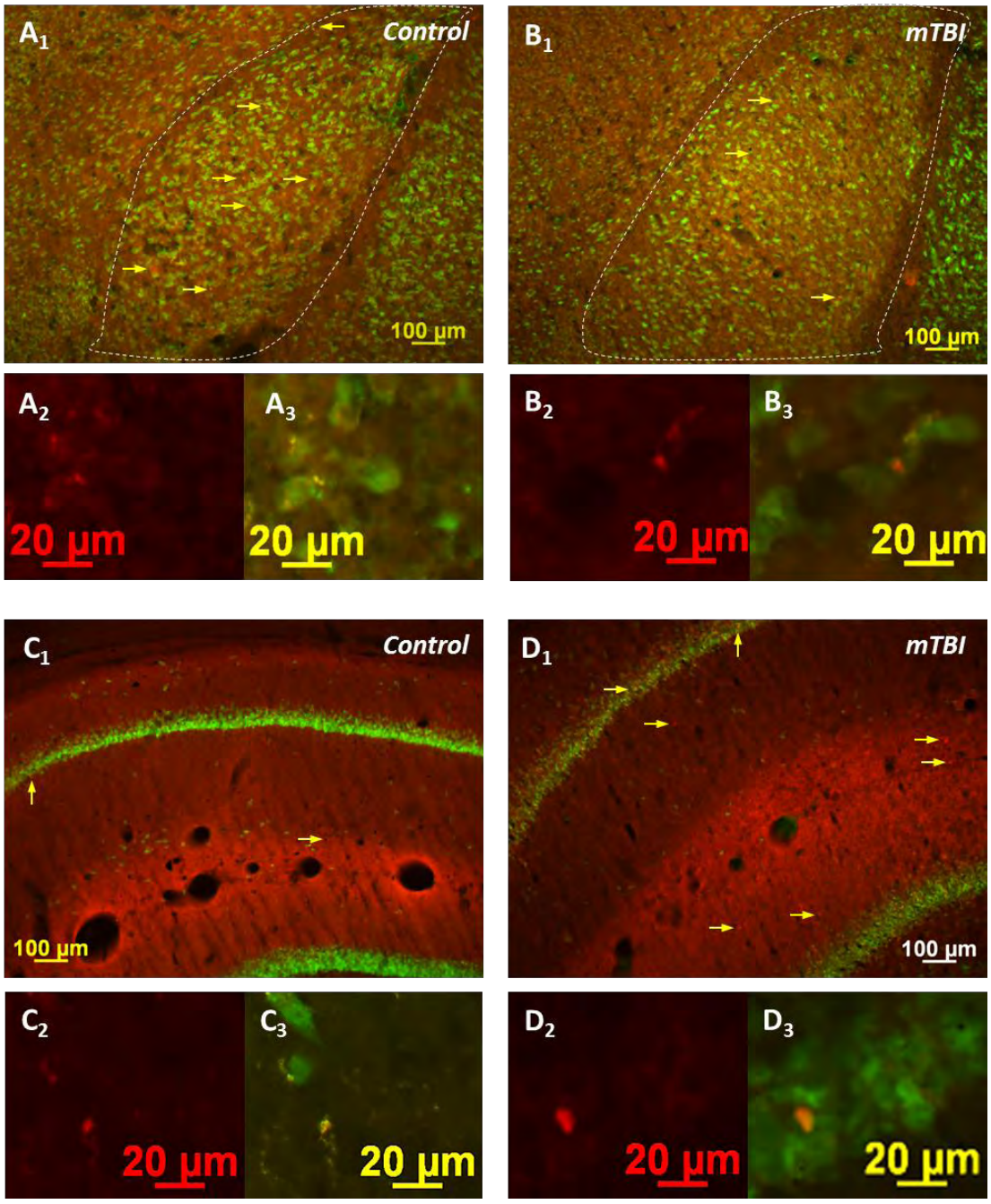

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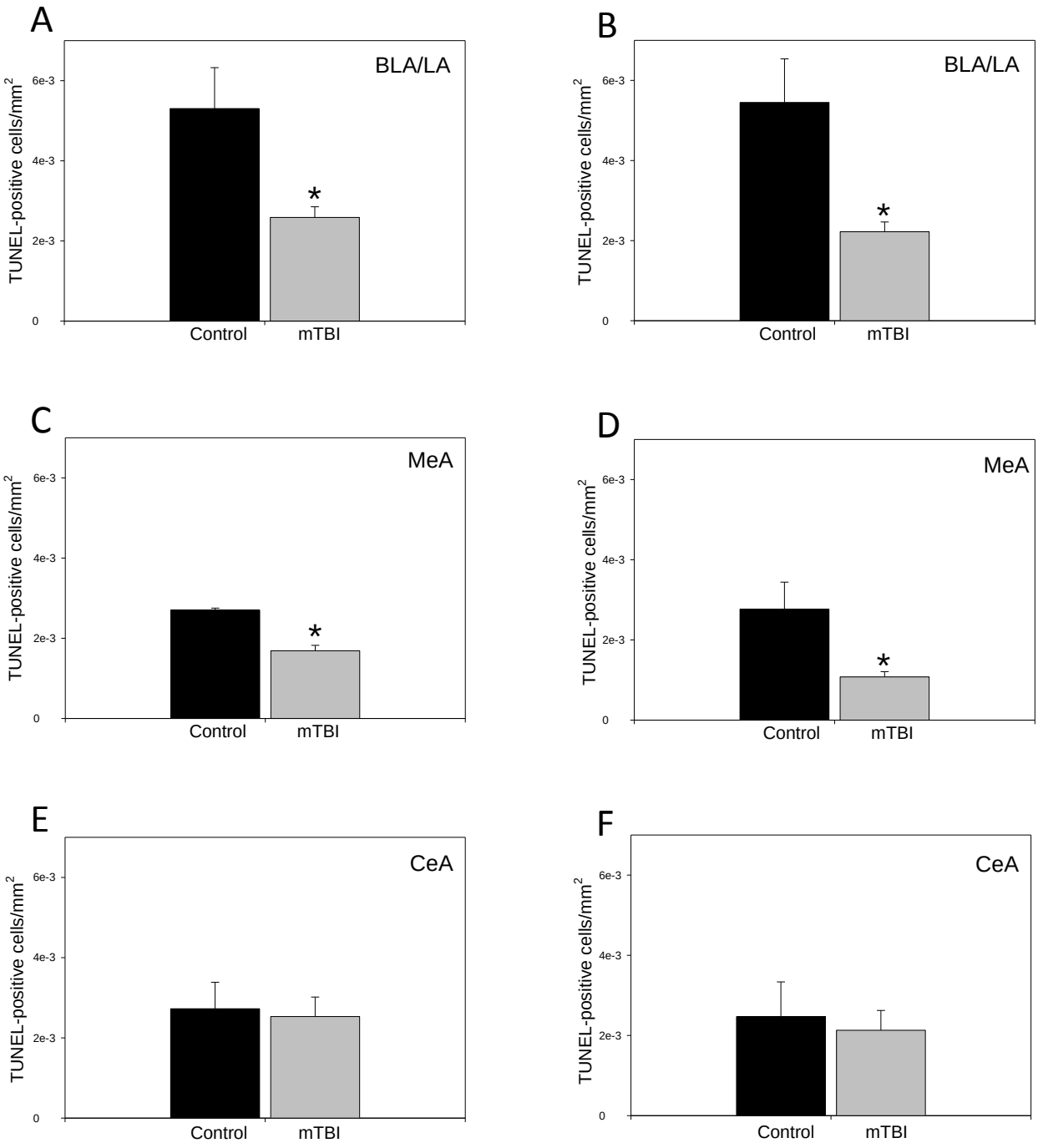

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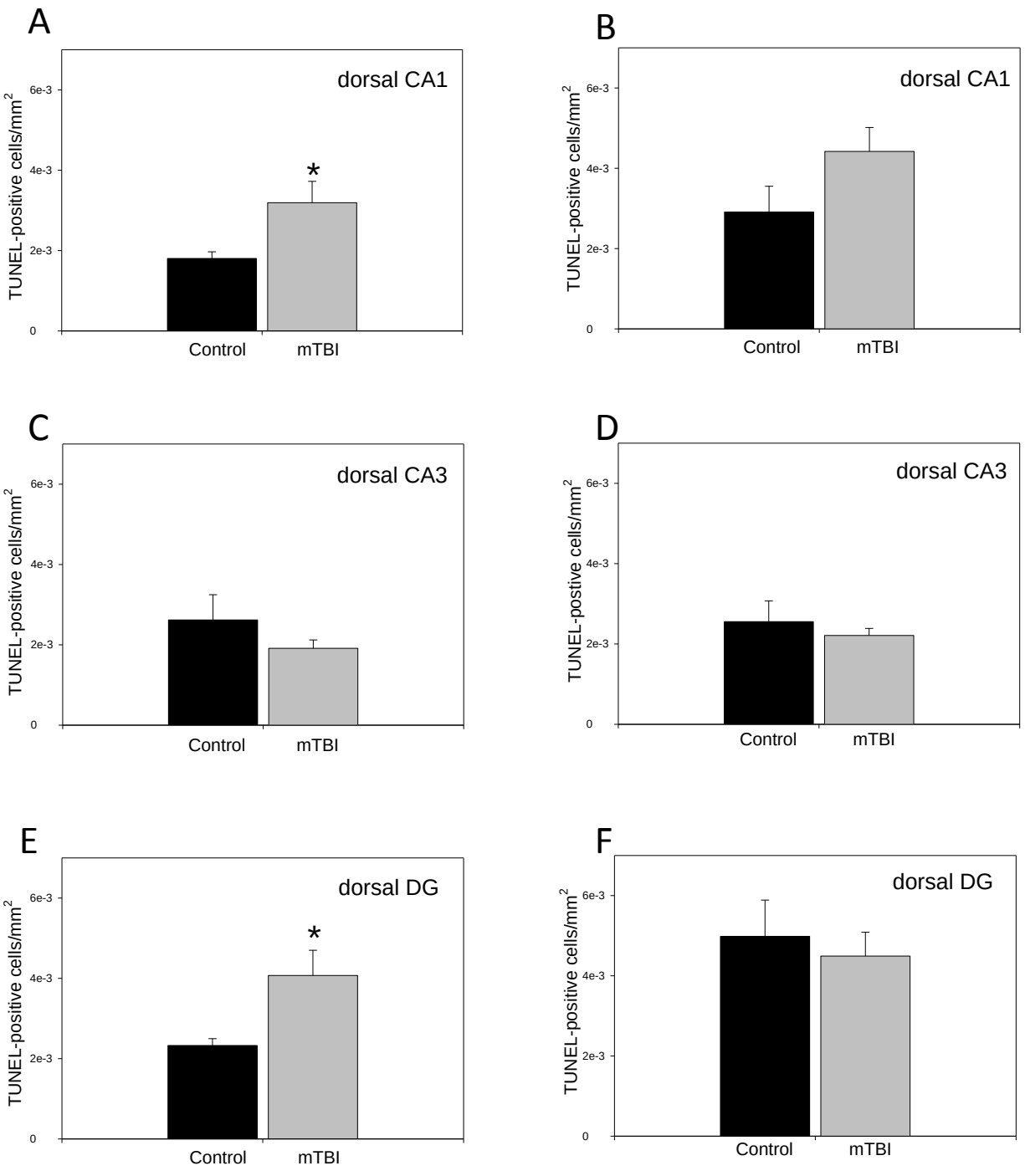

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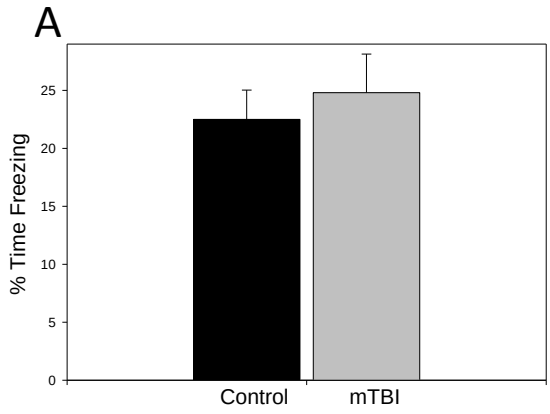

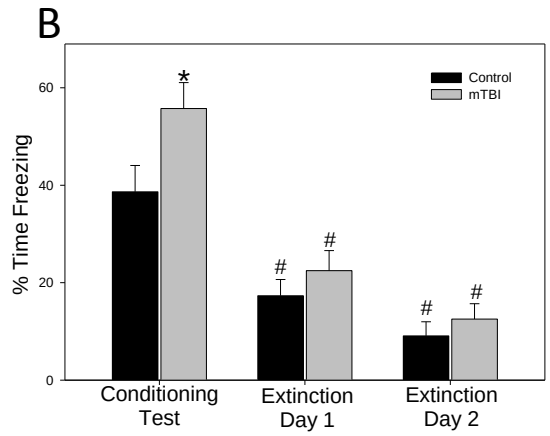

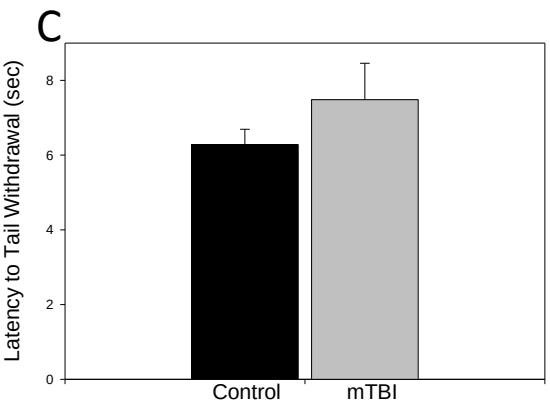

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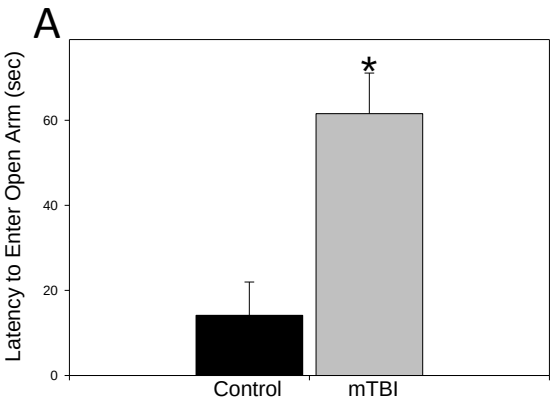

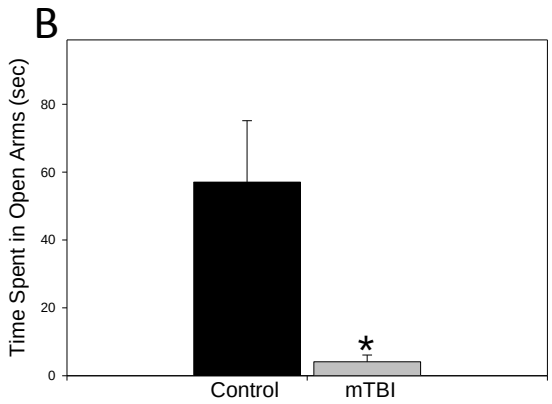

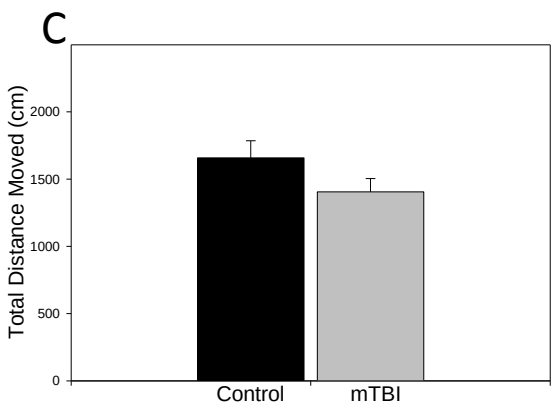

Meyer et al. Figure 9